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Enzymic Denitrosation of 1,3-Dimethyl-2-cyano-1-nitrosoguanidine in Rat Liver Cytosol and the Fate of the Immediate Product *S*-Nitrosoglutathione

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ABSTRACT. The tumorigenicity of certain *N*-nitrosoguanidinium compounds is limited, in rodents, by the propensity of these agents to be detoxified by denitrosation. Previous studies have revealed that rodent glutathione transferase isoenzymes are capable of catalyzing this process, generating exclusively the denitrosated guanidinium compound and *S*-nitrosoglutathione (GSNO). Experiments considering the denitrosation of 1,3-dimethyl-2-cyano-1-nitrosoguanidine (CyanoDMNG) in rat liver cytosol incubates are reported, with emphasis on the fate of GSNO. Incubates composed with equimolar CyanoDMNG and reduced glutathione (GSH) effected 100% denitrosation; the GSNO yield was less than expected as was the quantity of GSH consumed. When the anticipated 100% yield concentration of GSNO was applied to cytosol incubates, 20–40% of it rapidly disappeared. Nitrosated protein thiols accounted for 35% of the NO moiety released, nitrite ion 30%, and nitric oxide production was detectable. Concomitant with GSNO loss, GSH and oxidized glutathione (GSSG) were generated in yields similar to those detected in the CyanoDMNG/GSH incubates. Thus, the fate of GSNO in cytosol determines the yields of glutathione-based products, and the stoichiometry of the glutathione transferase reaction is demonstrated. In incubates composed with equimolar CyanoDMNG, GSH, and NADPH, denitrosation was again 100%, but GSNO yields were very low and residual GSH increased. Inclusion of NADPH in incubates containing the anticipated 100% yield concentration of GSNO resulted in rapid GSNO degradation, producing GSH and a detected but unidentified product; *S*-nitrosated protein, nitrite, and nitrate yields were minimal, nitric oxide production was abolished, and incubate response to a mercuric chloride/azo dye assay approached zero. The fate of the NO moiety consequent to this GSNO catabolism is presently unknown. BIOCHEM PHARMACOL 53;9:1279–1295, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nitrosoglutathione; *N*-nitrosoguanidinium compounds; denitrosation; glutathione transferase; rat liver cytosol; nitric oxide

NC† is the nitrosated derivative of cimetidine (Tagamet™), a very effective and orally administered histamine H_2-receptor antagonist utilized in the treatment of gastrointestinal disorders [1]. Interest in NC became keen when it was realized that the *in vivo* nitrosation of a portion of cimetidine in the stomachs of people ingesting the compound is a distinct possibility [2–4]. NC is an *N*-nitrosoguanidinium compound structurally similar to MNNG, a potent but locally acting carcinogen [5] (see Fig. 1). In common with other *N*-nitroso compounds and with potential carcinogens in general, MNNG and NC generate marked toxicity when applied to bacterial cells or to transformed mammalian cells in culture. Each is capable of promoting sister chromatid exchanges and chromosome aberrations, of inducing single-strand DNA breaks, DNA repair, and cell transformation, and each is mutagenic [6–11]. MNNG and NC are toxic to cells without the intermediary assistance of activating enzymes. Both are DNA methylating agents, generating the same O^6-methylguanine and 7-methylguanine lesions in DNA, at the same relative proportions, as the laboratory animal carcinogens 1-methyl-1-nitrosourea and dimethylnitrosamine [12–14].

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† *Abbreviations:* NC, nitrosocimetidine; MNNG, 1-methyl-2-nitro-1-nitrosoguanidine; CyanoDMNG, 1,3-dimethyl-2-cyano-1-nitrosoguanidine; CyanoDMG, 1,3-dimethyl-2-cyanoguanidine; GSNO, *S*-nitrosoglutathione; BN, *n*-butyl nitrite; BCNU, bis(2-chloroethyl)-nitrosourea; *p*-CMB, *p*-chloromercuribenzoate; RP-HPLC, reverse phase-high pressure liquid chromatography; GSSG, oxidized glutathione; SAX-HPLC, strong anion exchange-high pressure liquid chromatography; CM-DNP derivatives, *S*-carboxymethyl-*N*-2,4-dinitrophenyl derivatives; GSH, reduced glutathione; DTT, dithiothreitol; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); and ME, mercaptoethanol.

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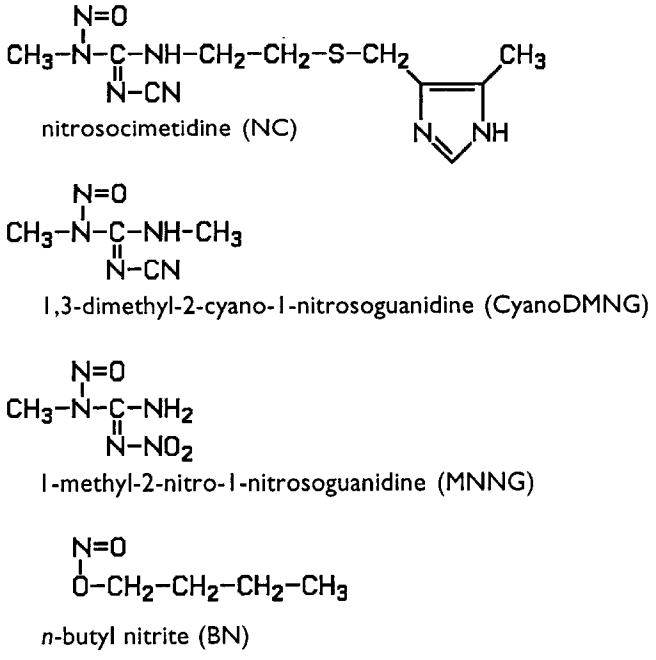

FIG. 1. Chemical structures of nitrosocimetidine (NC), 1,3-dimethyl-2-cyano-1-nitrosoguanidine (CyanoDMNG), 1-methyl-2-nitro-1-nitrosoguanidine (MNNG), and *n*-butyl nitrite (BN).

In spite of these telling *in vitro* properties, however, NC has been found to be a very weak or non-carcinogen when administered to rats or mice [15–18] and to be nonpathogenic to the rat at the highest practical doses [19]. It was soon recognized that the fate of dosed NC, at least in the rat and hamster, is essentially 100% denitrosation, a process that converts the potentially toxic compound to relatively innocuous cimetidine [14, 20]. In the hamster NC denitrosation is completed within the first 5 min after intravenous dosing [14]. Similarly, denitrosation of MNNG in the rat has been noted: 90% of the orally administered compound is recovered in the 24-hr urine as the denitrosated product 1-methyl-2-nitroguanidine, and only this compound is found in the circulating blood 20 min after dosing [5, 21].

We have discovered [22] that certain rat, mouse, and hamster glutathione transferase isoenzymes belonging to the mu class are capable of catalyzing the denitrosation of NC, MNNG, and the agent which we are developing as the archetype compound in this series of studies, CyanoDMNG (Fig. 1). Isoenzymes of the alpha and pi classes do not utilize these nitrosoguanidinium compounds as substrates [22]; the theta class of isoenzymes remains to be tested. The competent enzymes show strikingly high specific activities for the denitrosation process. The reaction products, produced with a 1:1 stoichiometry, are exclusively the denitrosated parent guanidinium compound, CyanoDMG in the CyanoDMNG case, and GSNO [22]. Our working hypothesis is that mu class glutathione transferase isoenzymes are responsible for the nitrosoguanidinium compound denitrosation process detected in rodent model systems.

There is limited and indirect evidence implicating glutathione transferase activities in the *in vivo* process. As noted, NC metabolism in the hamster is quite rapid and the outcome is 100% denitrosation. It has also been observed that depletion of the glutathione levels in the rat by pretreatment with diethyl maleate results in an 8-fold *increase*, relative to non-pretreated controls, in the liver DNA damage generated by intraperitoneally administered MNNG [23]. Similarly, intravenous administration of MNNG to diethyl maleate-pretreated hamsters effects a 10-fold increase in liver DNA methylation (7-methylguanine yield) relative to controls [24]. We conjecture that this increase in DNA damage reflects a decrease in glutathione transferase-dependent denitrosation. Seemingly at odds with the model that involves glutathione transferase is the observation that no detectable depletion of glutathione levels in the livers of hamsters 15 min after intravenous administration is evident even at the highest possible (solubility limited) doses of NC [24].

Studies were initiated to consider the catabolism of CyanoDMNG in the rat hepatocyte cultures. (It is noted here that CyanoDMNG is a preferred substrate in these investigations of the denitrosation phenomenon because, relative to MNNG, it is fairly resistant to nucleophilic attack at the central, guanidinium carbon [20, 22, 25, 26] and also because it lacks the data-confounding titratable proton present on the imidazole ring of NC (pK ~6.0 [20]). Preliminary experiments indicated that the hepatocyte preparations were capable of efficiently and quantitatively converting applied CyanoDMNG to CyanoDMG. However, it was noted that the cells could promptly generate up to 2-fold greater concentrations of CyanoDMG than there was intracellular glutathione available, without significant changes in the assessed glutathione levels. This observation prompted the present continuation of our earlier study of nitrosoguanidinium compound decomposition in rodent liver cytosol fraction [24], now with emphasis on evaluating the occurrence and the fate of the supposed glutathione transferase-generated product, GSNO.

We report that CyanoDMNG was quantitatively denitrosated when incubated with rat liver cytosol fraction in a glutathione-dependent reaction, that glutathione was consumed in the process, and that, consistent with glutathione transferase involvement, GSNO was generated in high yield. Evidence is presented that indicates that GSNO, in the absence of added NADPH, was involved in a dynamic, NO group exchange equilibrium with cytosolic protein thiols, and that small but readily detectable amounts of nitric oxide are released during this process. It is demonstrated as well that, in the presence of NADPH and cytosol fraction, introduced GSNO is quickly and efficiently converted to GSH and an as yet unidentified compound, together representing most of the input glutathione moiety. The production of nitric oxide is totally quenched when NADPH is present as is the yield of S-nitrosated protein; nitrite and nitrate are not products. Our results suggest that GSNO is actively catabolized by a cytosolic enzymic process that can

utilize NADPH as a cofactor. The fate of the NO moiety consequent to this process remains to be determined.

The companion paper presents evidence that CyanoDMNG processing by rat hepatocytes requires glutathione, involves glutathione transferase, and generates CyanoDMG at yields equal to that of the CyanoDMNG consumed [27]. Thus, while the production of intracellular GSNO seems likely, in fact the detected levels of this product are small and active catabolism is apparent. This indication that the introduced GSNO is catabolized may be of some significance in the biology of nitric oxide.

MATERIALS AND METHODS

Materials

CyanoDMG was generated from dimethyl *N*-cyanodithioiminocarbonate (Aldrich Chemical Co., Milwaukee, WI) following two rounds of ethanolic methylamine treatment as follows: 0.05 mol of the precursor was stirred overnight, at room temperature, with 0.055 mol of ethanolic methylamine (6.85 mL of 33% methylamine in absolute ethanol; Fluka Chemical Corp., Ronkonkoma, NY) plus 10 mL of absolute ethanol, and then held at −5° for several days. The resulting white crystalline precipitate was isolated and recrystallized once from 10 mL of absolute ethanol. A 0.005-mol portion of this intermediate {$CH_3SC(=NCN)NHCH_3$; 0.128 kDa} was then treated with 0.075 mol ethanolic methylamine (9.3 mL of 33% methylamine in absolute ethanol) in a sealed tube for 24 hr at 110°, cooled, and held at −5° overnight. The isolated crystals of CyanoDMG were recrystallized once from *n*-propanol (melting point, 174–176°; literature, 174–175° [28]). CyanoDMG was nitrosated to generate CyanoDMNG as previously described [2]. BN was purchased from the Aldrich Chemical Co. and used as supplied. GSNO was synthesized immediately prior to each experiment according to the procedure of Park [29]. Glutathione reductase (Type IV from baker's yeast; NAD[P]H:oxidized-glutathione oxidoreductase; EC 1.6.4.2), isocitric dehydrogenase (Type IV from porcine heart; isocitrate:$NADP^+$ oxidoreductase [decarboxylating]; EC 1.1.1.42), superoxide dismutase (from bovine liver; superoxide:superoxide oxidoreductase; EC 1.15.1.1), catalase (from bovine liver; H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6), and MNNG were purchased from the Sigma Chemical Co., St. Louis, MO. BCNU was from Bristol Laboratories, Oncology Products, Evansville, IN. All other chemicals were of reagent grade and were purchased from commercial sources. Female Sprague–Dawley rats (150–200 g) were obtained from Charles River Laboratories, Wilmington, MA.

Preparation of Rat Liver Cytosol Fraction/Liver Cytosol Incubates

Rats were fasted overnight prior to use. Rat liver cytosol fraction was prepared using Sucrose Buffer (0.25 M sucrose, 0.1 M Na_2HPO_4, pH 7.4) as previously described [24]. Prior to each set of experiments, low molecular weight compounds were removed from the isolated cytosol fraction by applying 3-mL aliquots of the crude preparation to Econo-Pac 10DG desalting columns (Bio-Rad Laboratories, Hercules, CA; 6.0 kDa exclusion limit), pre-equilibrated with Sucrose Buffer, and eluting with 4 mL of this same buffer. Cytosolic protein concentrations were routinely assessed using a modification of the Bradford dye-binding procedure [30] (Protein Assay Kit, Bio-Rad Laboratories, Richmond, CA) relative to BSA standards. The number of thiol groups in the prepared cytosol fraction available for reaction with *p*-CMB was determined as previously described [14] but in the present experiments by titrating the cytosol sample with the colorimetric reagent [31].

Cytosol fraction incubates composed in polypropylene test tubes were loosely capped and protected from ambient light. CyanoDMNG, CyanoDMG and MNNG were solubilized in DMSO and BN diluted with ethanol immediately before addition to the incubates; the final DMSO or ethanol in the incubates was 1%, v/v. GSNO synthesized in 0.05 M HCl [29] was pre-diluted with Sucrose Buffer and then added to the incubates; the final GSNO synthesis mixture dilution was 50-fold.

Preparation of BCNU-Modified Rat Liver Cytosol Fraction and Yeast Glutathione Reductase

Liver cytosol fraction and yeast glutathione reductase were pretreated with BCNU following a procedure similar to that described by Babson and Reed [32]. Preparations in Sucrose Buffer containing cytosol fraction at 27 mg protein/mL or 66.7 μM yeast glutathione reductase (calculated assuming a monomer molecular mass of 55 kDa [32]), 0.667 mM BCNU (delivered as a 32x stock prepared in acetone), and 1.34 mM NADPH (delivered as a 32x stock prepared in Sucrose Buffer) were incubated at 37° for 60 min at which time second aliquots of BCNU and NADPH were delivered to the preparations and incubation was continued for an additional 60 min. The incubates were then dialyzed extensively against Sucrose Buffer. Control preparations of cytosol fraction and of yeast glutathione reductase were similarly processed but not BCNU treated. The glutathione reductase activities of control and BCNU-treated preparations were determined by assessing the absorbance change at 340 nm as NADPH (100 μM) was consumed in the enzymic reduction of GSSG (1.00 mM), based on the method and using the buffer described by Babson and Reed [32].

CyanoDMNG and CyanoDMG Assays

CyanoDMNG and CyanoDMG were recovered from the various incubates by extraction into octanol using a methodology similar to that described previously [24] (in the present experiments, 0.50 mL of incubate sample was delivered into 1.00 mL *n*-octanol plus 0.50 mL water). Octanol extraction processing generally took about 90 sec. Iso-

lated phases queued for analysis were kept on ice. A portion of the octanol phase of these extracts (50 μL) was applied to a 8 × 100 mm μBondapac C-18/Radial-Pak HPLC column (RP-HPLC; Waters Chromatography Division, Millipore Corp., Milford, MA) and eluted at 3.0 mL/min (Rainin HPLC system, Rainin Instrument Co, Inc., Emeryville, CA) with a 4.0-min, linear, 10 mM K_2HPO_4, pH 7.0, 8–25% methanol gradient, followed by a 1-min 100% methanol wash to relieve the column of retained octanol and 5-min re-equilibration with the starting percentage of methanol. Elution peak areas, monitored at 214 nm, were computer integrated (Rainin Dynamax UV-1 Detector/HPLC Method Manager). The concentrations of CyanoDMNG and CyanoDMG in the various incubates were estimated by relating the integrated peak areas to those generated by similarly processed standard solutions of known solute concentrations.

GSNO, GSH, GSSG, NO_2^- and NO_3^- Assays

GSNO was recovered in the aqueous phase of incubate samples, octanol-extracted as described above, and was quantified, as specified and, depending on the experimental objective, by one or more of the three analytical HPLC methods noted below. Two of these methods allowed simultaneous quantitation of additional reaction products. It is noted that cytosolic protein is evidently denatured during the octanol/aqueous extraction procedure (enzymic reactions are stopped); much of the proteinaceous material from the incubate sample accumulates at the organic:aqueous interface.

RP-HPLC. GSNO has a retention time of 5.8 min on an RP-HPLC column equilibrated with 10 mM K_2HPO_4, pH 7.0, and eluted at a flow rate of 3.0 mL/min; 50 μL sample injection volume, elution profile monitored at 334 nm. The system was programmed to effect a 0–60% linear methanol gradient over min 4.5 to 6.5 of the elution process (to clear the column of other sample solutes), followed by 4 min of column re-equilibration with starting buffer.

SAX-HPLC. GSNO, GSSG, NO_2^- and NO_3^- in the aqueous phase of octanol extracts were resolved and quantified using a Whatman Partisil 10 SAX 25 cm HPLC cartridge column (SAX-HPLC; Whatman Inc., Clifton, NJ); flow rate 2.0 mL/min, sample injection volume 100 μL. The column was equilibrated with 7 mM H_3PO_4 (unadjusted pH 2.25) and the system programmed to effect a linear gradient to 300 mM H_3PO_4 (pH adjusted to 2.0 with KOH) between min 4.0 and 9.0 after sample injection and to return to the starting phosphoric acid concentration (6 min). The elution profile was monitored at 334 nm between min 0 and 2.4 (GSNO retention time, 2.2 min) and at 210 nm for the remainder of the chromatography run (GSSG, NO_2^-, and NO_3^- retention times 2.9, 4.9, and 9.6 min, respectively). In this methodology, the GSSG, NO_2^-, and NO_3^- yields were estimated by relating the integrated peak areas to those generated by similarly processed standard solutions of known solute concentrations. In the absence of a sufficiently precise independent measure of GSNO concentration, an estimate of the total "NO" units introduced to an incubate (GSNO plus NO_2^-) was based on the known concentration of sodium nitrite composing the GSNO synthesis mix; the initial, input GSNO concentration was calculated from this number and the observed (small) concentration of contaminating NO_2^-.

CM-DNP PROCEDURE/SAX-HPLC. In some experiments, aliquots of the aqueous phase of octanol extracts were first treated with iodoacetic acid to generate the *S*-carboxymethyl (CM) derivatives of solute compounds presenting free sulfhydryl groups and then treated with ethanolic 1-fluoro-2,4-dinitrobenzene to form the DNP derivatives [33]. Our protocol (CM-DNP Procedure) for producing these derivatives was essentially that described by Meredith [34]. GSNO is not destabilized by this process and *N*-DNP-GSNO is obtained. In this methodology, GSH and GSSG yield estimates were based on appropriately processed solutions of known solute concentrations. In the absence of a sufficiently precise independent measure of GSNO concentration, an estimate of the total glutathione units introduced to an incubate was based on the known concentration of glutathione composing the GSNO synthesis mix; the initial, input GSNO concentration was calculated from this number and the observed (small) concentrations of contaminating GSH and GSSG. Routinely, a parallel aliquot of sample was treated with 25 mM DTT for 1 hr, 37°, prior to the CM-DNP procedure. This treatment quantitatively converts GSNO as well as GSSG to GSH; the *N*-DNP–S–CM glutathione yield subsequently quantified represents the sum of these three compounds in the aqueous phase of the octanol extract. This processing was used to validate our input GSNO concentration estimates.

The CM-DNP derivatives were resolved by chromatography using anion exchange HPLC methodology similar to that developed by Reed *et al.* ([33]; CM-DNP Procedure/SAX-HPLC), utilizing as the aqueous elution solvent component the acetate buffer described (Acetate Buffer). Resolution was accomplished using a Whatman Partisil 10 SAX 25 cm HPLC cartridge column equilibrated with 10% Acetate Buffer in methanol and developed with a 4.0-min linear gradient to 30% Acetate Buffer in methanol starting at 4.0 min after sample injection (20 μL), followed by 8.0 min of isocratic 30% Acetate Buffer in methanol and finally 4.0 min of re-equilibration with 10% Acetate Buffer in methanol; flow rate of 2.0 mL/min, absorbance detection at 365 nm. The retention time for *N*-DNP–S–CM–glutathione was 12.2 min and the retention times relative to this standard for the other derivatized compounds considered were as follows: *N*-DNP-L-glycine, obscured by void volume absorbance; *N*-DNP-L-glutamic acid, 0.37; *N*-DNP-GSNO, 0.47; *N*-DNP-glutathione, 0.55; *N*-DNP-*S*-CM-L-cysteinylglycine, 0.73; *N*-DNP-*S*-CM-L-cysteine,

0.80; *N*-DNP-*S*-CM-glutathione, 1.00; *N*-DNP-γ-L-glutamyl-*S*-CM-L-cysteine, 1.18; *N,N*-bis-DNP-GSSG, 1.35.

Colorimetric GSH Assay

In some instances, the GSH concentrations in incubate samples, diluted at appropriate times to a final concentration of 5% TCA, were determined colorimetrically using the DTNB assay [35].

Protein S-Glutathiolation Assay

The degree of cytosolic protein S-glutathiolation was determined by first applying a 750-μL aliquot of an incubate sample to an Econo-Pac 10DG gel exclusion column (5°) and eluting with 4.0 mL of Sucrose Buffer (total processing time, 10 min). A 250-μL aliquot of this eluate was then treated with 10 mM ME for 10 min, 37°, to reduce any superficial protein-glutathione disulfides [36]. Controls were not ME treated. Protein was then precipitated with an equal volume of 10% TCA, and the glutathione yield in the recovered supernatant was determined using the CM-DNP Procedure/SAX-HPLC methodology described above. The data are normalized relative to the assessed protein concentration in the Econo-Pac 10DG column eluate.

Mercuric Chloride/Azo Dye Assay

An azo dye colorimetric assay was used to assess the total NO_2^- present in incubate samples and the NO_2^- generated in these samples after pretreatment with an estimated 20-fold excess of $HgCl_2$. Assessed were whole incubate samples or incubate samples first cleared of small molecules by passage through 10DG columns as described above (total processing time 10 min). Mercuric chloride liberates any NO moiety bound to protein [37], and that composing GSNO; this NO moiety is presumably then converted to nitrite ion. Immediately after constructing the $HgCl_2$ reaction mixtures (400 μL), 1.0 mL of a freshly prepared 1:1 mixture of 100 mM sulfanilamide and 1.0 mM *N*-(1-napthyl) ethylenediamine, each prepared in 3 M HCl, was added. The azo dye color yield developed in these mixtures was determined by absorbance at 546 nm [38]. Stock solutions of GSNO, which were found to generate the same degree of azo dye color as equimolar solutions of sodium nitrite, were used as concentration standards in these experiments. In the case of the protein-bound NO moiety, the data were normalized relative to the assessed protein concentrations in the 10DG column eluates.

Oxyhemoglobin to Methemoglobin Conversion Assay for Nitric Oxide

The quantity of nitric oxide generated in GSNO/cytosol fraction incubates was determined using a modification of the oxyhemoglobin to methemoglobin conversion assay described by Murphy *et al.* [39]. Human hemoglobin was isolated from peripheral whole blood using the method described by Waterman [40]. Hemoglobin was further purified by ion exchange chromatography [41, 42] (CM-Sephadex C50 column, 3.0 × 30 cm), developed using a 30-hr, linear gradient from pH 6.8 to 7.8, 0.01 M monobasic/dibasic sodium phosphate, 10 mL/hr. The peak 280 nm absorbance fractions were pooled and dialyzed against 50 mM K_2HPO_4, 0.5 mM Na_2EDTA, pH 7.0 (OxyBuffer). The spectral absorption characteristics of our preparations indicated that they were essentially 100% oxyhemoglobin [40]. The concentration of hemoglobin was assessed after conversion to the cyano form, assuming an extinction coefficient at 540 nm of 11,500 M^{-1} cm^{-1} per heme moiety [43].

Cytosol fraction was prepared for these experiments by passage through Econo-Pac 10DG desalting columns preequilibrated with OxyBuffer. Incubates for spectroscopy contained 100 μM oxyhemoglobin, an NADPH-regenerating system (10 mM trisodium isocitrate, 10 mM $MgCl_2$, 0.5 U/mL isocitric dehydrogenase), 64 U/mL superoxide dismutase, 100 U/mL catalase, ±1.0 mM NADPH and ± cytosol at 1 mg protein/mL in OxyBuffer. The reactions were initiated by adding GSNO stock to generate a starting incubate concentration of 100 μM (a 500-fold dilution of the GSNO synthesis mix). The time course of the oxyhemoglobin to methemoglobin conversion was evaluated by observing the change in absorbance at 577 vs 591 nm [39] (Perkin Elmer UV/Vis Lambda 2 Spectrometer) and assuming a $\Delta\epsilon$ at 577 nm of 8.9 mM^{-1} cm^{-1}, determined in the OxyBuffer system using standard methods [40, 43].

RESULTS

The rat liver cytosol preparations used in this work were cleared of low molecular weight compounds by passage through desalting columns characterized as excluding solutes greater than 6.0 kDa. Small molecule solutes were then added to cytosol incubates according to the experimental requirements.

Denitrosation of CyanoDMNG in Rat Liver Cytosol Incubates

CyanoDMNG, 1.00 mM in Sucrose Buffer, 37°, incubated with equimolar GSH and rat liver cytosol fraction (10.0 mg protein/mL) was converted rapidly and completely to its denitrosated derivative CyanoDMG (Fig. 2A). Rat liver cytosol contains significant quantities of the mu class glutathione transferase isoenzymes shown to be uniquely effective in catalyzing CyanoDMNG denitrosation and concomitantly producing equimolar yields of CyanoDMG and GSNO [22]. Even though the GSNO yield detected in the cytosol-mediated process was substantial, it represented only about 70% of the nitroso group removed from Cya-

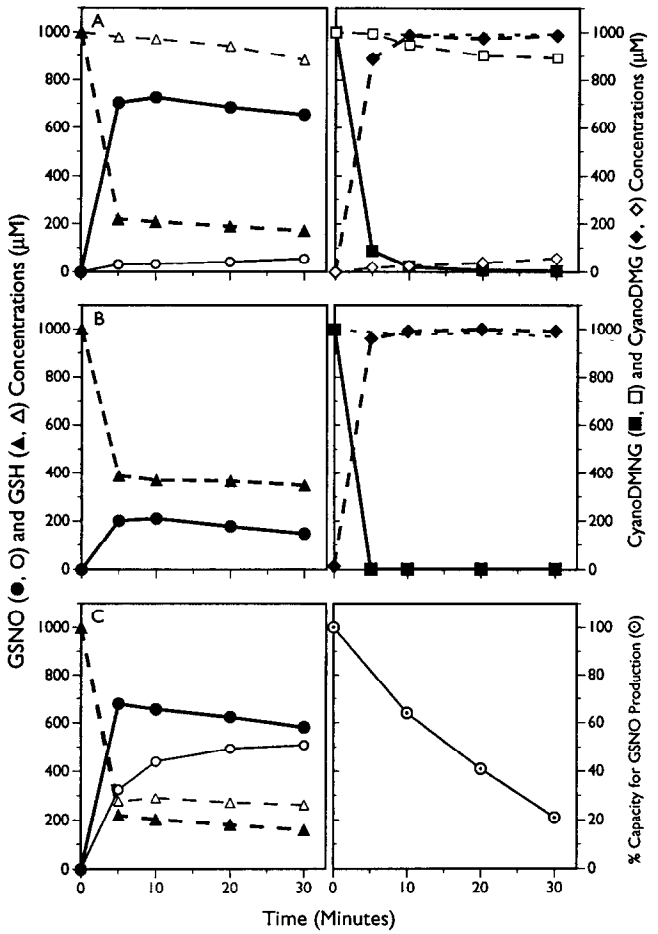

FIG. 2. GSH consumed (▲, △; DTNB assay) and the GSNO generated (●, ○; RP-HPLC assay) with time in incubates initially containing 1.00 mM CyanoDMNG (panels A and B) or 1.00 mM BN (panel C) with 1.00 mM GSH in Sucrose Buffer, 37°, in the presence (closed symbols; —/– –) or absence (open symbols; —/– –) of rat liver cytosol (10.0 mg protein/mL). In the experiment summarized in panel B, the incubate additionally included 1.0 mM NADPH. Also illustrated in panels A *right* and B *right* are the rates of disappearance of CyanoDMNG (■, □) and the appearance of CyanoDMG (◆, ◇) in these same incubates. The control shown in panel A, *right,* indicates the CyanoDMNG concentration with time when the nitroso compound was incubated in Sucrose Buffer, 37°, in the presence of cytosol fraction but in the absence of GSH (– – –); the data points are omitted for clarity. Similarly, the control shown in panel B, *right,* indicates the CyanoDMNG concentration with time when the nitroso compound was incubated with 1.0 mM NADPH (– – –); data points omitted. Panel C, *right,* indicates the percent capacity, relative to zero time, of sample aliquots withdrawn at the indicated times from a Sucrose Buffer incubate, 37°, containing 1.00 mM BN but no GSH or cytosol fraction, to generate GSNO in a subsequent incubate (nominally 0.9 mM BN, 0.9 mM GSH, 10 min, 37°; "0 time" GSNO yield = 350 μM). Each data point in this figure represents one quantitative assessment.

noDMNG. In addition, only 80% of the included GSH was consumed relative to the 100% denitrosated product yield (Fig. 2A). The sum of the GSNO and GSH concentrations in the illustrated experiment (GSSG yields were not assessed), relative to the input GSH concentration, ranged from 92% at the 5-min time point to 83% at 30 min.

Cytosol fraction incubates similarly composed with CyanoDMNG and GSH but containing in addition 1.0 mM NADPH also effected 100% CyanoDMNG denitrosation. In this case, the process generated an even lower apparent yield of GSNO, about 20% relative to the CyanoDMNG consumed. In addition, 40% of the input GSH was detected in the incubate after the denitrosation reaction was completed (Fig. 2B). The sum of the assessed GSNO and GSH concentrations relative to the input GSH concentration ranged from 50 to 59% between min 5 and 30.

Control experiments indicated that CyanoDMNG was essentially stable in incubates containing only liver cytosol fraction in Sucrose Buffer during the time frame of these experiments, and was stable as well in incubates containing only NADPH (Fig. 2, A and B, respectively). Inclusion of equimolar GSH in an incubate composed of CyanoDMNG in Sucrose Buffer resulted in some nitroso compound degradation, in 30 min representing about 10% of the input CyanoDMNG concentration (Fig. 2A). Approximately one-half of this cytosol-independent degradation was denitrosation, producing CyanoDMG and an equivalent amount of GSNO.

Denitrosation of BN in Rat Liver Cytosol Incubates

Recently reported spectrophotometric studies have been interpreted as indicating that rat liver cytosol fraction stimulates the production of GSNO in incubates containing the O-nitroso compound, BN, and glutathione [44]. It has also been demonstrated that the formation of GSNO in BN incubates containing GSH is catalyzed by cytosolic glutathione transferase isoenzymes [45] [in this case human isoenzymes of the alpha class (A1-1 and A2-2) and of the mu class (M1-1)]. To establish a relationship between these observations and the present experiments, the GSNO yield in rat liver cytosol incubates containing 1.00 mM BN and equimolar GSH was assessed. GSNO was generated in these incubates and it is clear that inclusion of the cytosol fraction in BN/GSH incubates enhanced its rate of production (Fig. 2C left panel). The yield of GSNO when cytosol fraction was included and the level of GSH remaining in the incubate were identical to those detected in the CyanoDMNG experiment described above (Fig. 2A). The sum of the GSNO and GSH concentrations in this experiment, relative to the input GSH concentration, ranged from 99% at the 5-min time point to 83% at 30 min. As previously noted [45], and in contrast to the CyanoDMNG case, substantial interaction between GSH and BN generated GSNO in cytosol-free incubates (Fig. 2C left panel). Under our conditions, this process was 50% complete in 4 min, and after 30 min the sum of the GSNO and GSH recovered in the incubate represented 77% of the input GSH concentration. BN incubated alone in Sucrose Buffer degraded with an estimated half-life of 15 min, 37° (Fig. 2C right panel).

GSNO Decomposition in Cytosol Incubates as a Function of Included NADPH: Overview

The observed changes in the GSNO concentration as a function of time, and as a function of included NADPH, when incubated in rat liver cytosol preparations, as well as the yields of several of the products generated in these incubates are summarized in the series of experiments illustrated in Fig. 3. A number of other experiments detailing specific aspects of the GSNO degradation phenomenon are described in subsequent sections.

GSNO was observed to be stable over the 30-min incubation period in the absence of cytosol fraction (Fig. 3, panel B1). Addition of GSNO to an incubate containing rat liver cytosol fraction, however, resulted in a partial but rapid loss of the GSNO, a portion of which was converted to GSH (panel B2). In the experiment presented, 20% of the input GSNO had disappeared by the 5-min time point, about 50% of which had been converted to GSH. In our experience, the magnitude of this rapid depletion of GSNO from cytosol incubates varied with the cytosol preparation and was generally in the 20–40% range of the input GSNO (1.00 mM GSNO, 10.0 mg cytosolic protein/ mL). At the 30-min time point in the illustrated GSNO/cytosol fraction experiment (Fig. 3, panel A2) the assays were able to account for essentially all of the glutathione units applied as GSNO: 70% remained as GSNO, 20% was GSH, and 10% was GSSG (in all cases GSSG concentration is expressed in glutathione units). A substantial fraction (25%) of the NO moiety applied to the incubates as GSNO was not accounted for by the residual GSNO, NO_2^- and NO_3^- yields.

The yield distribution of GSNO and GSH in cytosol incubate (Fig. 3, panel B2) was similar to that described above for the degradation of CyanoDMNG or BN in the presence of GSH and cytosol fraction (but in the absence of NADPH), suggesting that the yields of these compounds detected in the CyanoDMNG and BN experiments (Fig. 2, A and C) reflected the subsequent disposition of the GSNO product.

Inclusion of NADPH in incubates composed of GSNO and liver cytosol fraction resulted in a marked and rapid loss of GSNO (Fig. 3, A and B, panels 3–5). As higher concentrations of NADPH were included, the fraction of input glutathione units recovered as GSH increased. Nevertheless, a substantial fraction of the input was lost from our assays (30% in the 2.0 mM NADPH case). In concert with this, the NO moiety introduced as composing GSNO failed to reappear as NO_2^- or NO_3^-. Control experiments indicated that GSNO was relatively stable in the presence of 1.00 mM NADPH alone (Fig. 3, panel B4).

Most of the experiments described below consider the 0- to 10-min time frame, which contains the rapid phase of GSNO degradation apparent in Fig. 3 and which was appropriate to our analytical procedures. The continued changes in product yields with time subsequent to the 5-min time point evident when NADPH was limiting or not present will be discussed.

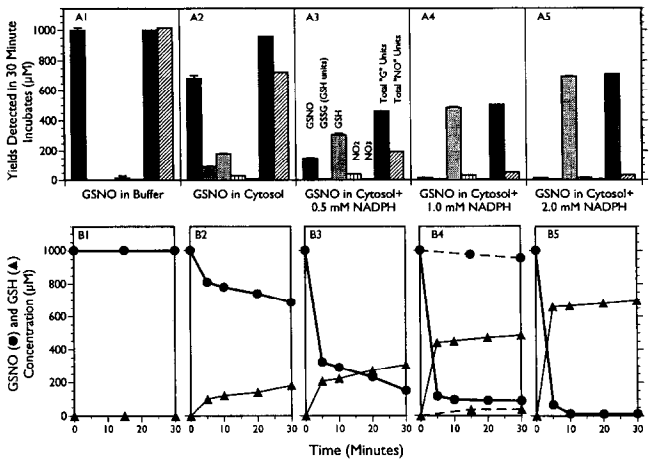

FIG. 3. Concentration of GSNO (initially 1.00 mM) in Sucrose Buffer with time, 37°, in the absence or presence of rat liver cytosol (10.0 mg protein/mL) in incubates containing the indicated amounts of added NADPH. The upper panels show the GSNO, GSSG (expressed in glutathione units), NO_2^- and NO_3^- concentrations (SAX-HPLC), and the GSH concentration (DTNB assay) remaining 30 min after addition of GSNO. The shaded bars in these panels represent the mean ± SD, N = 3. Also indicated are the sum of the concentrations of the assessed glutathione-containing compounds (GSNO + GSH + GSSG = Total "G" Units) and the sum of the assessed "NO"-containing compounds (GSNO + NO_2^- + NO_3^- = Total "NO" Units) based on these several assays. The lower panels show the change in GSNO (●; RP-HPLC) and GSH (▲; DTNB assay) concentrations in these same incubates with time. Also shown in panel B4 is the change with time of the GSNO and GSH concentrations when GSNO was incubated in Sucrose Buffer containing 1.0 mM NADPH (– – –). In the lower panels, each point represents one quantitative assessment.

Fate of the Glutathione Moiety in GSNO/Cytosol Incubates

ASSESSMENT OF CYTOSOLIC PROTEIN S-GLUTATHIOLATION. Cytosol incubates of varying composition were prepared as noted in Table 1 and, at 10 min after adding GSNO, aliquots were withdrawn and assessed for cytosolic protein S-glutathiolation as described in Materials and Methods. The data summarized in Table 1 indicate that the absolute yield of ME-sensitive glutathione-protein mixed disulfides was maximal when 1.0 mM NADPH was present in the incubate. However, the greatest yield of protein S-glutathiolation relative to the GSNO which had disappeared from the various incubates occurred under the condition of no added NADPH and was estimated to be 3%. Although this GSNO-based formation of mixed disulfides may represent an important process in the biological function of the compound, the yield was insignificant in our present accounting. Adding NADPH to the incubates was seen to significantly erode the yields of glutathione-protein mixed disulfides relative to the GSNO decomposed.

REDISTRIBUTION OF THE GSNO GLUTATHIONE MOIETY AMONG SMALL MOLECULE SPECIES. The CM-DNP Procedure/SAX-HPLC methodology permitted a simultaneous

TABLE 1. GSNO concentration in whole incubates (μM) and apparent protein S-glutathiolation (μM GSH bound/10 mg protein) after 10 min, 37°, Sucrose Buffer, in incubates initially containing 1.00 mM GSNO ± rat liver cytosol fraction at 10.0 mg protein/mL and various concentrations of NADPH

	RP-HPLC GSNO†	CM-DNP procedure/SAX-HPLC* GSH released from protein	
		-ME treatment	+ME treatment‡
Cytosol alone		BDL§	BDL
GSNO alone	1000 ± 17‖ (0)		
GSNO + Cytosol	768 ± 25 (232)	BDL	6.9 ± 1.1 [3.0]
GSNO + Cytosol + 0.5 mM NADPH	357 (643)	BDL	9.3 [1.4]
GSNO + Cytosol + 1.0 mM NADPH	77 ± 6 (923)	BDL	13.1 [1.4]
GSNO + Cytosol + 2.0 mM NADPH	0 (1000)	BDL	6.7 [0.7]
GSNO + Cytosol + 5.0 mM NADPH	0 (1000)	BDL	6.2 [0.6]

* Appropriately derivatized GSH solutions of known solute concentration were used as concentration standards.

† The GSNO concentration in the "GSNO alone" incubates was assigned a value of 1000 μM based on the assumption of 100% yield in the synthesis mix. The GSNO yields under the several other incubate conditions were estimated relative to this assumed input GSNO value. In parentheses: the apparent μM GSNO lost from the incubates.

‡ In brackets: percentage of GSNO lost from the incubates evidently recovered as S-glutathiolated cytosolic protein.

§ BDL: below detectable levels.

‖ Mean ± SD, N = 3.

assessment of GSNO, GSH, and GSSG yields recovered from an incubate sample. Consistent with the experiments illustrated in Fig. 3, the data summarized in Table 2 indicate that application of GSNO to a cytosol incubate ("GSNO + Cytosol") resulted in significant loss of the GSNO with the concomitant production of GSH and GSSG. In this experiment, essentially all of the glutathione units (96%, data column G) can be accounted for as GSNO, GSH, or GSSG; the +DTT data (102% yield, data column H) corroborates this finding.

Also consistent with the experiments summarized in Fig. 3 are the data indicating that including NADPH in cytosol incubates promoted rapid GSNO degradation to generate high yields of GSH (Table 2, data column C), and that only a fraction of the glutathione units applied as GSNO was recovered as GSNO, GSH, and GSSG in these incubates (the sum indicated in data column G). Pretreatment of the incubate samples with DTT verified this apparent loss of glutathione units (data column H).

The CM-DNP Procedure/SAX-HPLC methodology revealed another compound, identified in Table 2 data column E and in the Fig. 4 chromatogram as "Unknown," which apparently represents a major product or intermediate generated in the NADPH-promoted GSNO degradation process. Detection of this product as a dinitrophenyl derivative suggests that it is likely to possess a primary amino group. As a first approximation, it is assumed that the extinction coefficient (365 nm) for the DNP-derivative of this unknown is similar to that of *N*-DNP–*S*-CM–glutathione, and that the DNP-reaction yield is quantitative (both conditions which are experimentally met in the GSNO case). Thus, it is calculated that, in this particular experiment, 85–95% of the GSNO decomposed in the cytosol-mediated degradation process promoted by included NADPH was recovered either as GSH or as this unknown compound (Table 2, data column F). In our several experiments, the relative yield of GSH and unknown compound generated in similarly composed incubates was seen to vary with the cytosol preparation; however, the total glutathione units recovered as these entities was always greater than 80%.

Experiments which considered the production of GSH and the unknown compound as a function of time in GSNO/cytosol incubates containing NADPH indicated that, within the time resolution of our methodology, the two products were generated coincidentally with GSNO degradation (Fig. 5, A and B). The fate of the unknown compound as it steadily disappeared from our incubates with time, as illustrated, for example, in Fig. 5B, also remains to be determined. It is known that this rate of disappearance is not enhanced in incubates containing 5 mM NADPH. (It is noted that the unknown compound is stabilized by DNP derivatization.)

Based on the relative retention times of appropriately derivatized authentic markers (CM-DNP Procedure/SAX-HPLC), it is evident that the unknown compound is not L-cysteine, L-glutamic acid, L-cysteinyl-L-glycine, γ-L-glutamyl-L-cysteine, $NADP^+$, or NADPH. The retention time of the unknown is insensitive to carboxymethylation (±iodoacetic acid in the CM-DNP Procedure), indicating that the presence of a free sulfhydryl group is unlikely. Also, the HPLC retention time of the unknown product and its yield were insensitive to DTT treatment before derivatization, providing evidence that the unknown is unlikely to be an *S*-nitrosothiol compound or a reducible disulfide. The unknown compound was somewhat acid labile and was lost after 1 hr, 5°, if the incubate sample was made 5% in TCA or 10% in perchloric acid. It is nearly quantitatively recovered from solution in 5 mM acetic acid, pH 3.5, 30 min, 5° (conditions required for our evaluation

TABLE 2. Concentrations (μM) of solutes containing the glutathione moiety after 10 min, 37°, Sucrose Buffer, in incubates initially containing 1.00 mM GSNO ± rat liver cytosol fraction at 10.0 mg protein/mL and various concentrations of NADPH

	RP-HPLC	CM-DNP procedure/SAX-HPLC (-DTT)*						CM-DNP procedure/ SAX-HPLC (+DTT)†
	A	B	C	D	E	F	G	H
	GSNO	GSNO	GSH	GSSG (expressed in glutathione units)	Unknown‡	Sum: GSNO + GSH + GSSG + Unknown (B + C + D + E)	Sum: GSNO + GSH + GSSG (B + C + D)	GSH
GSNO alone§	904 ± 20‖	904 ± 14	72 ± 7	24 ± 2	BDL¶	1000	1000	1016 ± 11
GSNO + Cytosol	560 ± 17	567 ± 17	267 ± 11	127 ± 20	BDL	961	961	1016 ± 6
GSNO + Cytosol + 0.5 mM NADPH	122	111	381	16	345	853	508	519
GSNO + Cytosol + 1.0 mM NADPH	19 ± 7	13 ± 3	466 ± 9	BDL	407 ± 20	886	479	463 ± 23
GSNO + Cytosol + 2.0 mM NADPH	BDL	BDL	549	BDL	380	929	549	520
GSNO + Cytosol + 5.0 mM NADPH	BDL	BDL	560	BDL	393	953	560	580

* The GSH and GSSG yields were calculated relative to appropriately derivatized GSH and GSSG solutions of known solute concentration. The total glutathione moiety introduced to the incubates is set equal to 1000 μM ("GSNO alone" data column G) based on the known glutathione concentration composing the GSNO synthesis mix. The GSNO present in the "GSNO alone" incubates (data column B) is thus an estimation based on the known concentration of GSH used to synthesize the GSNO stock solution and the levels of contaminating GSH and GSSG assessed in these preparations using the CM-DNP Procedure/SAX-HPLC (-DTT) methodology. The GSNO yields under the several other incubate conditions were estimated relative to this assumed input GSNO value. This same GSNO input value was also used as a basis for the RP-HPLC data calculations.

† Appropriately derivatized GSH solutions of known solute concentration were used as standards.

‡ The yield of the unknown product was estimated using an assumed extinction coefficient derived from our analysis of *N*-DNP–*S*-CM–glutathione stocks of known concentration.

§ "GSNO alone" data points were gathered at 0 min.

‖ Mean ± SD, N = 3.

¶ BDL: below detectable levels.

of the unknown product yield in primary rat hepatocytes [27]).

Utilizing the CM-DNP Procedure/SAX-HPLC methodology, we found that 10-min incubates containing GSH, cytosol fraction, and either CyanoDMNG, BN, or MNNG contained nearly the same yields of GSNO, GSH, and GSSG as observed in incubates composed of GSNO and cytosol fraction (Table 3). Inclusion of NADPH in parallel incubates as they were composed resulted in significantly lower GSNO yields with concomitant increases in recovered GSH and the unknown product.

Fate of the NO Moiety Derived from GSNO in GSNO/Cytosol Incubates

Aliquots were withdrawn from cytosol incubates 10 min after adding GSNO and cleared of small molecules by rapid passage through 10DG desalting columns. Samples of column eluates were then treated with mercuric chloride to release any thiol-bound NO from the recovered macromolecules and subsequently submitted to the azo dye colorimetric assay. In these same experiments, other incubate aliquots were evaluated for total incubate azo dye-sensitive material (i.e. not 10DG-column processed) using the mercuric chloride/azo dye method as well as for quantifying GSNO, NO_2^- and NO_3^- yields using SAX-HPLC. The gathered data are presented in Table 4.

PRODUCTION OF NO_2^- AND NO_3^- IN GSNO/CYTOSOL INCUBATES. About 28% of the GSNO lost from 10-min GSNO + Cytosol incubates, relative to GSNO alone incubates, was represented as NO_2^- (Table 4; data column B). This percentage recovery approximated that observed for GSSG in similarly composed cytosol incubates, 31% relative to GSNO alone incubates (Table 2, data column D). Including NADPH in GSNO/cytosol incubates was seen to erode the NO_2^- yields significantly such that in the presence of 2.0 mM NADPH and when GSNO was completely degraded, the recovery of this anion was at background levels.

The data listed in Table 4, data column C, indicate that conversion of GSNO-derived NO moieties to NO_3^- does not appear to be a factor under any of the several incubate conditions.

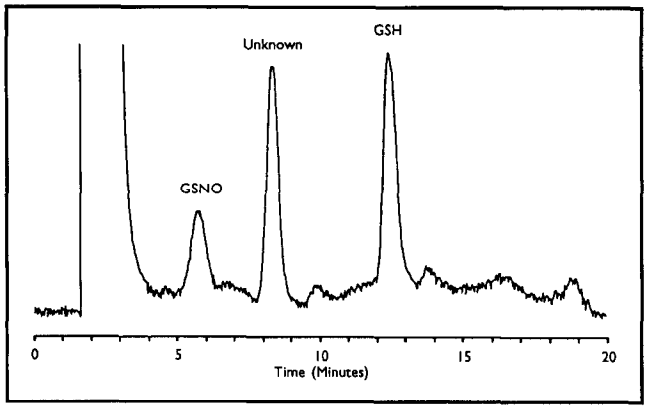

FIG. 4. CM-DMP Procedure/SAX-HPLC chromatogram of a processed incubate sample (10 min, 37°) that initially contained 1.00 mM GSNO, 0.5 mM NADPH, and cytosol fraction at 10.0 mg protein/mL. The retention times of the indicated elution peaks, relative to *N*-DNP-S-CM-glutathione (1.00) were 0.46 for *N*-DNP-GSNO and 0.67 for the DNP derivative of the Unknown.

NO-EXCHANGE EQUILIBRIUM BETWEEN GLUTATHIONE AND CYTOSOLIC PROTEIN THIOLS IN GSNO INCUBATES IN THE ABSENCE OF NADPH. GSNO is considered to be an NO^+ carrier, capable of exchanging the nitroso moiety with appropriate nucleophiles [46], including available protein thiols and thiolate-containing small molecules in solution; in many such examples, the exchange equilibrium is quickly established [29, 47–50]. After GSNO was incubated with cytosol fraction for 10 min, about 35% of the nitroso moiety released from GSNO was recovered as constituting nitrosated protein thiols (Table 4, "GSNO + Cytosol", data column E). The yield of cytosolic protein thiol-bound NO moiety was 11.8 nmol/mg protein. It is noted that when the individual product assessments of what are expected to be the $HgCl_2$/azo dye-sensitive materials in the GSNO + Cytosol incubates were summed (GSNO + NO_2^- + protein-bound NO moiety; Table 4, date column H), the result agrees with what was assessed in intact incubates to within 10%.

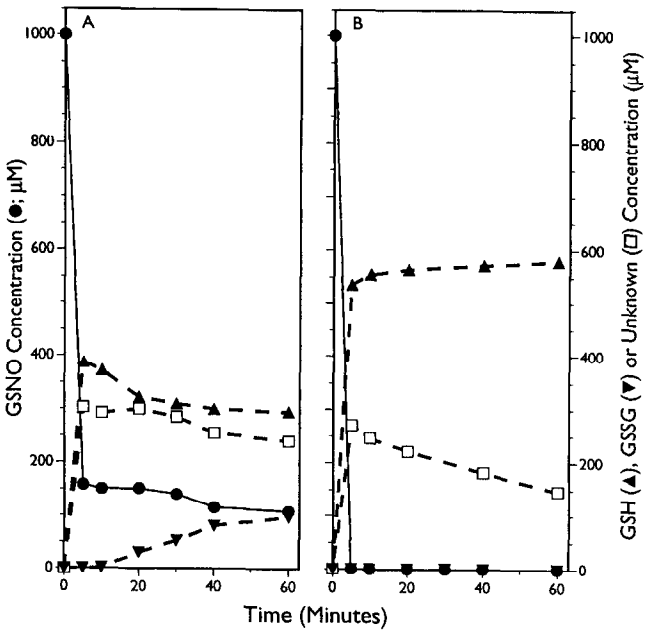

FIG. 5. Concentration of GSNO (●; initially 1.00 mM) in Sucrose Buffer, 37°, and the observed yields of GSH (▲), GSSG (▼) and unknown (□) determined using CM-DMP Procedure/SAX HPLC and as a function of time, in incubates initially containing 1.0 mM (panel A) or 2.0 mM (panel B) NADPH and cytosol fraction at 10.0 mg protein/mL. Each point in this figure represents one quantitative assessment.

If there is indeed a dynamic NO-exchange equilibrium established in our GSNO + cytosol incubates between glutathione and available protein thiols, added GSH in the incubate as it is constituted would be expected to shift this equilibrium towards increased intact GSNO. As illustrated in Fig. 6A, in an incubate without added GSH a 360 μM decrease in the GSNO concentration was detected within the first 10 min. In a parallel cytosol incubate that initially included 2.0 mM GSH in addition to GSNO, the GSNO decrease assessed at 10 min was 300 μM. Correspondingly, when the concentration of GSNO remaining in a series of 10-min cytosol fraction incubates was evaluated as a function of included GSH (Fig. 6B), an apparent increase in GSNO stability was observed such that with 10.0 mM included GSH the GSNO loss was only 210 μM. The GSNO yield data in Fig. 6B describe a plot that is linear with the logarithm of GSH concentration (panel B, inset); thus, additional significant increases in apparent GSNO stability would require somewhat greater input GSH. If it is envisioned that inclusion of 10 mM GSH shifted the presumed equilibrium wholly in favor of GSNO, then it can be estimated that 40% of the GSNO concentration diminution observed in the exogenous GSH-free incubates was due to reversible transfer of the NO moiety to protein thiols. This is equivalent to 14.4 nmol of protein thiol-bound NO moiety/mg of cytosolic protein.

Titrations of native cytosolic protein using *p*-CMB indicated 120 ± 10 nmol of accessible thiol/mg protein (mean ± SD, N = 3). Therefore, approximately 10% of the protein thiol groups became nitrosated when 1.0 mM GSNO was incubated with 10 mg protein/mL cytosol fraction.

LOSS OF ASSESSABLE GSNO-DERIVED NO MOIETY IN CYTOSOL INCUBATES CONTAINING NADPH. A spectral quenching artifact was apparent in the $HgCl_2$/azo dye assay when NADPH was present. This is evidenced by comparing the SAX-HPLC data in Table 4, data column D, indicating a 5% decrease in GSNO concentration when incubated with 2.0 mM NADPH (no cytosol) relative to "GSNO Alone," with the data in Table 4, data column G where a nearly 25% decrease was indicated in the parallel colorimetric assay. A similar NADPH-dependent decrease in colorimetric response was detected when NO_2^- solutions were evaluated (data not shown). Consequently, in those cases in which NADPH was present, the colorimetric de-

TABLE 3. Solute concentrations (μM) after 10 min, 37°, Sucrose Buffer, in incubates initially containing 1.00 mM GSNO, CyanoDMNG, BN or MNNG, 1.00 mM GSH, rat liver cytosol fraction at 10.0 mg protein/mL and ± 1.00 mM NADPH

	CM-DNP procedure/SAX HPLC (-DTT)*				
	A	B	C	D	E
	GSNO	GSH	GSSG (expressed in glutathione units)	Unknown†	Sum: GSNO + GSH + GSSG + Unknown (A + B + C + D)
GSNO alone‡	957 ± 11§	33 ± 4	10 ± 2		1000
GSNO + Cytosol	634 ± 59	248 ± 31	152 ± 5	13 ± 10	1047
GSNO + Cytosol + NADPH	90 ± 10	626 ± 45	5 ± 1	96 ± 3	817
CyanoDMNG + Cytosol + GSH	642 ± 45	163 ± 2	85 ± 6	19 ± 6	909
CyanoDMNG + Cytosol + GSH + NADPH	135 ± 14	475 ± 49	18 ± 2	149 ± 5	777
BN + Cytosol + GSH	555 ± 30	252 ± 9	92 ± 19	29 ± 25	928
BN + Cytosol + GSH + NADPH	59 ± 16	619 ± 60	4 ± 3	163 ± 11	845
MNNG + Cytosol + GSH	525 ± 19	317 ± 47	74 ± 13	10 ± 4	926
MNNG + Cytosol + GSH + NADPH	23 ± 7	659 ± 26	5 ± 1	135 ± 3	822

* The GSH and GSSG yields were calculated relative to appropriately derivatized GSH and GSSG solutions of known solute concentration. The total glutathione moiety introduced to the incubates was set equal to 1000 μM ("GSNO alone," data column E) based on the known glutathione concentration composing the GSNO synthesis mix. The GSNO present in the "GSNO alone" incubates (data column A) is thus an estimation based on the known concentration of GSH used to synthesize the GSNO stock solution and the levels of contaminating GSH and GSSG assessed in these preparations using the CM-DNP Procedure/SAX HPLC (-DTT) methodology. The GSNO yields under the several other incubate conditions were estimated relative to this assumed input GSNO value.

† The yield of the unknown product was based on an assumed extinction coefficient derived from our analysis of *N*-DNP–*S*-CM–glutathione stocks of known concentration.

‡ "GSNO alone" data points were gathered at 0 min.

§ Mean ± SD, N = 3.

termination of total incubate azo dye-sensitive NO moiety (Table 4, data column F) becomes quantitatively unreliable. Nevertheless, it is evident that inclusion of NADPH in GSNO/cytosol incubates caused significant decreases in the total azo dye responsive material present in the incubates, beyond what might be due to the quenching artifact (Table 4, compare data column F with data column G). Within 10 min of adding GSNO to incubates containing 2.0 mM NADPH, only 3% of the NO moiety was detectable by our assays. Parallel decreases were detected as well

TABLE 4. Concentrations (μM) of solutes containing the NO moiety after 10 min, 37°, Sucrose Buffer, in incubates initially containing 1.00 mM GSNO ± rat liver cytosol fraction at 10.0 mg protein/mL and various concentrations of NADPH

	SAX-HPLC*				Mercuric chloride/Azo dye†			
	A	B	C	D	E	F	G	H
	GSNO	NO_2^-	NO_3^-	Control: GSNO/No cytosol	Protein-bound NO (μM/10 mg protein)	Total incubate azo dye-sensitive material	Control: total incubate azo dye-sensitive material/ no cytosol	Calculated azo dye-sensitive material (A + B + E)
Cytosol alone		BDL‡	11 ± 5§		BDL	BDL		BDL
GSNO alone	983 ± 18	17 ± 5	19 ± 2	983 ± 18		1000 ± 11	1000 ± 9	1000
GSNO + Cytosol	640 ± 11	114 ± 3	22 ± 2		118 ± 2	950 ± 12		872
GSNO + Cytosol + 0.5 mM NADPH	173 ± 10	106 ± 2	25 ± 1	992 ± 4	93 ± 1	601 ± 11	937 ± 9	372
GSNO + Cytosol + 1.0 mM NADPH	11 ± 5	57 ± 4	24 ± 1	972 ± 8	53 ± 1	315 ± 2	874 ± 4	120
GSNO + Cytosol + 2.0 mM NADPH	BDL	23 ± 1	15 ± 1	945 ± 4	5 ± 1	31 ± 2	771 ± 8	28

* The NO_2^- and NO_3^- yields were calculated relative to chromatogram peak areas generated by similarly processed samples of known solute concentration. The GSNO concentration in the "GSNO alone" incubates is an estimation based on the known concentration of sodium nitrite used to synthesize the GSNO stock solution and the levels of contaminating nitrite assessed in these preparations using the SAX-HPLC methodology. The GSNO yields under the several other incubate conditions were estimated relative to this assumed input GSNO value.

† The "GSNO alone" incubate was used as the concentration standard in these assays and, based on the sodium nitrite included in the GSNO synthesis mix, was assigned a value of 1000 μM.

‡ BDL: below detectable levels.

§ Mean ± SD, N = 3.

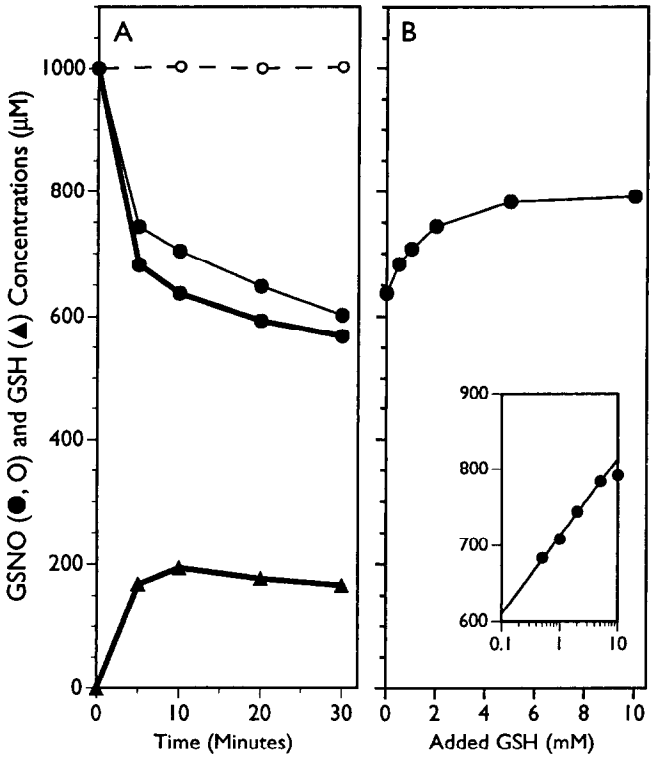

FIG. 6. (A) Concentration of GSNO (●, ○ initially 1.00 mM) in Sucrose Buffer with time, 37°, in the absence (open symbols, dashed line) or presence (closed symbols, solid lines) of dialyzed rat liver cytosol (10.0 mg protein/mL), and including no GSH (▬) or 2.0 mM GSH (—). Also shown is the GSH generated (▲) as GSNO disappeared from the incubate, which included no exogenous GSH at the outset. (B) Concentration of GSNO (●) detected after 10 min of incubation, 37°, in the presence of cytosol fraction (10.0 mg protein/mL) and various concentrations of included GSH. Each point in panels A and B represents one quantitative assessment. *Inset:* data of Panel B as a semilog plot; the logarithmic line drawn through the data does not include the 10 mM result.

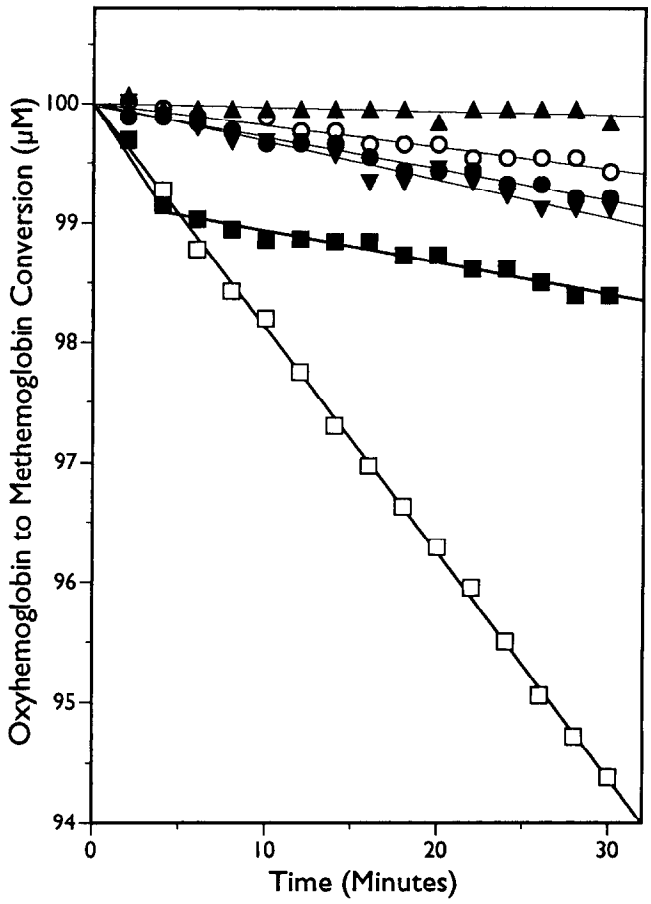

FIG. 7. Production of nitric oxide as determined by the rate of conversion of 100 μM oxyhemoglobin to methemoglobin, 30°, in OxyBuffer. Indicated is the conversion generated by 100 μM GSNO in the presence of 1.0 mg protein/mL rat liver cytosol (□), and also in incubates containing, in addition, 1.0 mM NADPH (■). Controls are oxyhemoglobin incubates containing GSNO alone (○), GSNO plus NADPH (▼) and NADPH alone (▲). Also shown is the rate of conversion generated by 100 μM GSNO in the presence of 100 μM GSH (●). Spectral scans were initiated within 1 min of adding GSNO to the incubates and in all cases early time absorbencies extrapolated back to the oxyhemoglobin zero time control values. Each data point in this figure represents one quantitative assessment.

in the independently evaluated nitrite ion and in the protein thiol-bound NO moiety yields (data columns B and E), both of which contribute to the total incubate azo dye-sensitive material.

ASSESSMENT OF THE NITRIC OXIDE GENERATED IN GSNO/CYTOSOL INCUBATES ± NADPH. The oxyhemoglobin to methemoglobin conversion assay indicated an apparent steady release of nitric oxide in GSNO/cytosol incubates throughout the 30 min observation period (Fig. 7). The characteristic rapid decrease in GSNO concentration upon application to cytosol incubates was not reflected in these data. The rate of methemoglobin production in GSNO/cytosol incubates was 10-fold greater than that detected in GSNO incubates without included cytosol; nevertheless, the yield of methemoglobin was relatively small, equivalent to 6% of the input GSNO during 30 min. It is possible that the NO-exchange equilibrium between glutathione and protein thiols is established rapidly and that the detected nitric oxide represents a chance release during this process. However, when GSNO was incubated with equimolar GSH in this assay, minimal nitric oxide release was apparent (Fig. 7) in spite of the expectation that NO moiety exchange among the glutathione thiols was occurring.

When GSNO was added to a cytosol/oxyhemoglobin incubate that contained NADPH as well, the oxyhemoglobin to methemoglobin conversion was quickly suppressed (Fig. 7). Nitric oxide is evidently not a product of the cytosol-mediated degradation of GSNO promoted by NADPH. Also, this experiment demonstrated that the process mediating the formation of methemoglobin when GSNO is in-

cubated with cytosol fraction is quickly undone by including NADPH.

DEMONSTRATION OF THE LIKELY NON-INVOLVEMENT OF GLUTATHIONE REDUCTASE IN CYTOSOL-DEPENDENT GSNO DECOMPOSITION. Substantial NADPH-dependent glutathione reductase is present in the liver cytosol fraction. It is conceivable that GSNO interacts with glutathione reductase, passing its NO moiety to an active site thiolate anion with the concomitant liberation of GSH. We determined that a cytosol preparation diluted to 1.0 mg protein/mL contained glutathione reductase activity equivalent to that of 11.7 pmol of purified yeast glutathione reductase/mL. Our experiment contrasted the stability of 1.0 mM GSNO in the presence of 1.0 mM NADPH in this cytosol preparation with that in a parallel incubate containing 11.7 pmol of purified yeast glutathione reductase/mL. Evaluated in this same experiment were cytosol fraction and yeast glutathione reductase preparations that had been treated with BCNU, an agent that irreversibly inhibits glutathione reductase upon carbamoylating the active site cysteine residues [32, 51]. The glutathione reductase activities of the BCNU-treated liver cytosol and yeast glutathione reductase preparations were 2 and 3%, respectively, of the untreated control values.

Although there was some loss of intact GSNO with time in the presence of yeast glutathione reductase and NADPH, which was diminished by about 50% in the BCNU-treated preparation (Fig. 8), GSNO was substantially and more rapidly consumed in the NADPH/cytosol preparation. BCNU-treated cytosol demonstrated a slightly enhanced capacity to degrade GSNO.

In a related experiment (data not shown), it was determined that sample aliquots taken at intervals over 30 min from a co-incubation of yeast glutathione reductase (13.3 pmol enzyme protein/mL) with NADPH (1.0 mM) and GSNO (1.0 mM), 37°, demonstrated the same glutathione reductase activity as did samples drawn from a parallel incubate containing only glutathione reductase and NADPH. Thus, any interactions between GSNO and the enzyme in the incubate evidently did not serve to inhibit the enzymic activity.

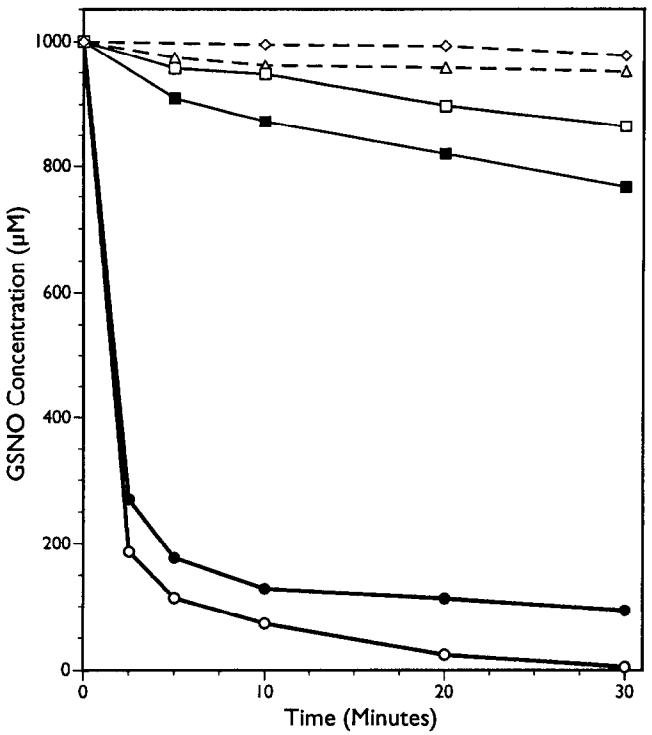

FIG. 8. GSNO concentration as a function of time (initially 1.0 mM) in incubates prepared with 1.0 mM NADPH and either rat liver cytosol at 1.0 mg protein/mL (●; assessed glutathione reductase activity equivalent to 11.7 pmol yeast glutathione reductase/mL) or yeast glutathione reductase at 11.7 pmol enzyme protein/mL (■), 37°. Also illustrated are the analogous experiments using rat liver cytosol (○) and yeast glutathione reductase (□) that had been pretreated with BCNU; the treated preparations had lost 98 and 97% of their glutathione reductase activity relative to non-treated controls, respectively. Controls are GSNO alone in Sucrose Buffer (◇), and GSNO plus cytosol at 1.0 mg protein/mL (△).

DISCUSSION

The observed glutathione-dependent, rat liver cytosol-mediated denitrosation of CyanoDMNG is in concert with our earlier results which considered NC and MNNG denitrosation mediated by hamster liver cytosol [24]. In that work it was established that the process is enzymic, that it could be thwarted by a number of glutathione transferase inhibitors, and that the quantities of nitroso compound consumed and denitrosated product generated were equivalent and approximated the reduced glutathione utilized. It was subsequently discovered that certain members of the mu class of cytosolic glutathione transferase isoenzymes isolated from laboratory rodents are uniquely capable and very efficient at catalyzing nitrosoguanidinium compound denitrosation. The products of this enzymic process are the denitrosated guanidinium compound and GSNO, generated in a 1:1 ratio [22].

The present CyanoDMNG experiments identified GSNO as a major product of the glutathione-dependent denitrosation reaction in liver cytosol incubates. The concentrations of GSNO generated were less than that expected based on the quantity of CyanoDMNG consumed and of CyanoDMG produced, and GSH and GSSG were identified as coproducts. However the GSNO, GSH, and GSSG concentrations produced in the CyanoDMNG reaction were found to be nearly identical to those detected when the anticipated 100% yield concentration of GSNO was incubated in cytosol fraction. Thus, the stoichiometry of the glutathione transferase-mediated process is demonstrated, the glutathione-based product yields reflecting the fate of GSNO.

GSNO is relatively stable in neutral buffer solution. Nei-

ther GSH nor GSSG is generated at significant rates when GSNO is incubated alone in solution, and little GSSG is produced when GSNO is incubated with an excess of GSH.* However, when GSNO was introduced to a cytosol incubate (which was first cleared of small molecules by passage through a 6.0-kDa gel exclusion column), there was a rapid decrease in the concentration of intact GSNO (20–40% in our experiments) with the concomitant production of GSH, GSSG, and *S*-glutathiolated cytosolic protein, representing 60, 30, and 3%, respectively, of the GSNO lost from a 10-min incubate. We were able to account for essentially all of the input glutathione units. Nitrite ion was generated in GSNO/cytosol incubates at yields nearly equivalent to the GSSG produced; nitrate was not a product. About 35% of the nitroso group lost from the applied concentration of GSNO was recovered as protein thiol-bound NO moiety. A significant fraction of the NO moiety released from GSNO (35–40%) remained unaccounted for. Nevertheless, our detected yields of GSNO, NO_2^-, and protein thiol-bound NO moiety represented 87% of the anticipated azo dye-sensitive material (after $HgCl_2$ treatment) applied to the incubates as GSNO (with some contaminating nitrate) and the intact GSNO/cytosol 10-min incubates yielded 95% of the input azo dye-sensitive material.

Experimental results from this and other laboratories have suggested that GSNO is capable of participating in dynamic NO-exchange with protein and small molecule thiols: equilibrium is accomplished rapidly and little NO moiety is lost from the exchange participants [29, 47–50]. We view it as likely that the rapid drop in the GSNO concentration when it is applied to cytosol incubates is due to the establishment of NO exchange with cytosolic protein thiols. Detection of $HgCl_2$-sensitive protein-bound NO moiety attests to this as does our observation that inclusion of 10 mM GSH in the cytosol incubate as it was composed resulted in a 40% increase in the recovered GSNO yield; in this case, the protein thiol/GSH NO-exchange equilibrium was evidently shifted to favor GSNO.

The results from the oxyhemoglobin to methemoglobin conversion assay indicate that nitric oxide is generated in our GSNO/cytosol incubates in perhaps modest yields (in our experimental protocol the methemoglobin generated represented about 6% of the input GSNO over 30 min). Nevertheless, the rate of nitric oxide production in these incubates was 10-fold greater than that detected when GSNO and oxyhemoglobin were incubated in the absence of added cytosol or when GSNO and GSH were coincubated with oxyhemoglobin. In addition, methemoglobin was generated in GSNO/cytosol incubates at a seemingly linear fashion, proceeding from 100% oxyhemoglobin at zero time. We surmise that the supposed NO-exchange equilibrium involving input GSNO and cytosolic protein thiols is rapidly established, and that nitric oxide production derives from it, perhaps reflecting a chance nitric oxide release during the transfer reaction.

The formation of GSSG and nitrite in our incubates is possibly related to this nitric oxide production. The formation of these products effectively drain glutathione units and NO moiety from the proposed dynamic NO-exchange equilibrium. This depletion of the exchange system may be evidenced by the steady loss of GSNO from cytosol incubates observed over time.

When NADPH was included in a cytosol fraction incubate, the applied GSNO was quickly dispatched. The oxyhemoglobin to methemoglobin conversion capacity characteristic of GSNO/cytosol incubates was quenched, indicating at once that the supposed NO-exchange reaction ceased to exist and that nitric oxide was not a product of NADPH-promoted GSNO degradation. In the presence of NADPH, the nitrite yield derived from GSNO in cytosol incubates was reduced to background levels and the assessed *S*-nitrosated cytosolic protein yield approached zero. Nitrate levels remained close to background. Within 10 min of composing a GSNO/cytosol incubate with excess NADPH, all of the material that was sensitive to the mercuric chloride/azo dye assay had vanished. The fate of the NO moiety is presently being determined. Most of the input glutathione moiety was recovered from these incubates as GSH and an unidentified but major coproduct (detected as a DNP derivative) which we are now in the process of isolating for identification.

We have recently isolated and are now characterizing a cytosolic enzyme from rat liver which catalyzes GSNO degradation (designated as GSNO terminase).* The purified enzyme reflects the characteristics noted for the cytosolic activity except that it has become apparent that the glutathione-based enzymic reaction products are predominately GSSG and the unidentified coproduct. The GSH yields generated in GSNO/cytosol incubates containing NADPH are evidently derived in large measure from cytosolic NADPH-dependent glutathione reductase operating on GSSG. We have also learned that GSNO terminase will utilize either NADPH or NADH as cosubstrate.

These findings may have important implications for the biological actions of nitric oxide. It has been shown that glutathione readily combines with the autoxidation products of nitric oxide (nitrogen oxide intermediates, "NO_x" [52–54]); the immediate and relatively stable product is GSNO. Further, it has been proposed that GSNO may function as a reservoir of the NO moiety and a donor of the various redox forms of nitric oxide ($NO^\bullet$, NO^+, NO^-) to effect biological function [46, 50, 52, 55]. The destruction of GSNO promoted by cytosolic GSNO terminase possibly represents the mechanism by which the activities of nitric oxide and its redox forms as signal transduction devices are truncated and also a mechanism by which cells are protected from the toxicity of NO moiety excess.

* Jensen DE and Belka GK, manuscript in preparation.

* Jensen DE and Belka GK, manuscript in preparation.

In the accompanying article [27], we describe our studies which considered the catabolism of CyanoDMNG mediated by rat hepatocytes in primary culture and the concomitant production of GSNO. It is noted that the *N*-nitroso compounds CyanoDMNG and MNNG and the *O*-nitroso compound BN are quite similar in that when each was incubated in rat liver cytosol preparation fortified with equimolar GSH (1.0 mM), the yields of GSNO, GSH, and GSSG produced in the incubates were essentially identical and equivalent to those detected when 1.0 mM GSNO was applied to a cytosol incubate. The result indicates that GSNO is the immediate and exclusive glutathione-derived product. Evidently the alpha and mu class glutathione transferase isoenzymes requisite for catalyzing the denitrosation reactions are well represented in rat liver cytosol fraction. However, in the continuing study of denitrosation and the fate of GSNO, CyanoDMNG has the technical advantage of stability. CyanoDMNG has a half-life estimated to be in the range of 1 week in neutral pH buffer, 37°; MNNG about 3 hr [20]; and BN approximately 15 min. In the non-enzymic interaction with equimolar GSH (1.0 mM, neutral pH, 37°), CyanoDMNG has a half-life of about 3 hr (50% denitrosation), BN has a half-life of 4 min, and MNNG is totally dispatched in less than 5 min (5% denitrosation) [20].

Finally, it is emphasized that glutathione transferase activity toward CyanoDMNG has been found to be restricted to certain members of the mu class of isoenzymes, well represented in hamster, mouse, and rat liver [22] and present in detectable and variable amounts in other rat tissues [56]. Pi class glutathione transferase isoenzymes (human† and rodent [22]), generally found to be quantitatively predominant in transformed cell lines and in tumor cells [57–62], do not catalyze CyanoDMNG denitrosation. Alpha class isoenzymes (human† and rodent [22]) also do not utilize this compound as a substrate. In addition, CyanoDMNG denitrosation is not catalyzed by the well studied, polymorphic, human mu class isoenzymes GSTM1-1 [62] variably found in human cell lines and tumorous tissue [63]. In all cases considered thus far (rodent), glutathione transferase isoenzyme activity and specificity towards NC parallel that towards CyanoDMNG [22]. The observed vulnerability of established cell lines to the genotoxic and cytotoxic effects of NC may well be due to the predominance of denitrosation-incompetent glutathione transferase isoenzymes in these cell types.

Only human mu class glutathione transferase isoenzyme GSTM2-2 has been identified as competent in the CyanoDMNG denitrosation reaction (four mu class isoenzymes have been tested [62]). As observed in the case of the several denitrosation-competent rodent mu class enzymes, GSTM2-2 has a remarkably high specific activity for the CyanoDMNG substrate. It can perhaps be anticipated that NC too will be found to be a GSTM2-2 substrate. The GSTM2-2 isoenzyme is found in skeletal muscle, aorta, and heart but not, evidently, in human liver [64–66]. Consequently, humans may not have the same degree of protection against NC toxicity (first pass denitrosation) as is apparent in laboratory rodents.

† Mannervik B, Berhane K and Jensen DE, unpublished observations.

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